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Factors Influencing Spawning and Pairing in the Scale Worm *Harmothoe imbricata* (Annelida: Polychaeta)

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Abstract. Endocrine and environmental factors control reproduction of the polynoid scale worm *Harmothoe imbricata.* We confirmed that the rate of vitellogenesis was greater in winter specimens transferred from ambient regimes of photoperiod and temperature to a light:dark (LD) photoperiod of 16:8 at 10°C and showed that the number of females spawning was significantly greater than for those transferred to LD8:16 at 10°C. The endocrine mediation of this response was investigated using prostomium implantations. Significantly more LD8:16 females implanted with prostomia from LD16:8 conditioned females spawned than LD8:16 females implanted with LD8:16 prostomia. Females without prostomia failed to spawn. LD16:8 exposure may increase levels of a possible "spawning hormone" in the prostomium. Spawning proceeded in these LD16:8 females and allowed spawning to occur in LD8:16 females implanted with LD16:8 prostomia. In LD8:16 prostomia, titers of the spawning hormone reached the threshold in significantly fewer individuals, so that significantly fewer females implanted with LD8:16 prostomia spawned.

Using Y-maze choice chambers, pair formation was shown to be under pheromonal control, with males being attracted to mature females but not to females carrying fertilized oocytes or to LD8:16 conditioned females. Production of this attraction pheromone can, therefore, be manipulated through photoperiodic control, suggesting a link between oogenesis, spawning, and pheromone production.

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Introduction

The polynoid scale worm *Harmothoe imbricata* is a common inhabitant of temperate intertidal rocky shores, where it lives under rocks and small stones. It is an active carnivore that preys upon other small invertebrates. The reproductive biology of this species is relatively well understood. It is a dioecious, iteroparous species with an annual cycle of reproduction (Daly, 1972, 1974; Garwood, 1980). Females develop two cohorts of oocytes: the first is grown slowly during the winter months to be spawned in March; the second is produced rapidly and spawned about 30 days after the first. During the breeding season individuals pair, a behavior in which a male lies closely along the dorsal surface of the female (Daly, 1972). Cohorts of oocytes, once spawned, are fertilized and carried under the female's dorsal elytra (scales) during embryogenesis. Embryos are released as trochophores after about 16 days.

Oogenesis is initiated in late September without any specific environmental input. During the autumn, the first cohort grows under conditions of decreasing environmental temperature, and growth is accelerated by exposure to low temperatures (Garwood, 1980). The stabilization of oocyte development also requires exposure to a light-dark (LD) cycle with a photophase less than 13 h for between 42 and 55 days during the late autumn period. If these photoperiods are not experienced, oocyte development is aborted (Clark, 1988). Once the winter solstice has passed, natural populations respond to a second photoperiodic input. An exposure to LD cycles with a photophase above 10 or 11 h increases oocyte growth rate and may synchronize oogenesis and spawning among individuals (Garwood and Olive, 1982; Clark, 1988).

Although environmental manipulation affects both oogenesis and spawning, the endocrine role in mediating these

influences has received comparatively little attention. Bentley *et al.* (1994) and Lawrence (1996) confirmed the presence of a gonadotrophic hormone from the prostomium that probably mediates oogenesis, and it seems likely that the hormone is secreted only under the appropriate regimes of photoperiod and temperature. Nevertheless, the role of the endocrine system in spawning is unknown in this species. In a number of other polychaete species, the implantation or injection of prostomia into individuals has been used to investigate their endocrine control of reproduction and spawning (Golding, 1983; Bentley *et al.*, 1984; Pacey and Bentley, 1992; Bentley *et al.*, 1994; Watson *et al.*, 2000). In the work reported here, transplantation experiments establish the role of the endocrine system in mediating photoperiodic influences, and environmental manipulation is used to investigate the effect of photoperiod on spawning and oogenesis of the first cohort of oocytes.

Pheromones coordinate and control reproduction in a number of marine invertebrates, including species of the opisthobranch mollusc *Aplysia* (Zeeck *et al.*, 1990, 1996; Painter *et al.*, 1998), by inducing spawning and by attracting other individuals towards spawning animals. In this paper we provide evidence of an attraction pheromone produced by female *Harmothoe imbricata* to attract males for mating, and we examine the influence of environmental manipulation on its production.

The results presented here provide the first link between environmental conditioning, endocrine activity, and pheromonal control of attraction for pairing and spawning in a polychaete species. *Harmothoe imbricata* is thus likely to become a model species for the investigation of environmental-endocrine-pheromone interactions.

Materials, Methods, and Results

Photoperiodic control of oocyte growth and spawning mediated by the endocrine system

Collection and maintenance of animals. Specimens of *Harmothoe imbricata* were collected from the intertidal zone of three rocky shores in E. Scotland, UK: St Andrews (56°20′N, 2°47′W), Kingsbarns (56°18′N, 2°38′W) and Fife Ness (56°16′N, 2°35′W). Individuals were maintained in glass crystallizing dishes containing 100 ml of TFSW (twice-filtered seawater, pore size 0.34 μm) and provided with a cleaned *Patella vulgata* shell for shelter. All worms were hand-fed pieces of *Mytilus edulis* muscle once per week, after which the water was changed. All individuals were collected in December and January and kept at 10°C and ambient photoperiods until the experiments commenced on 9 February 1999.

To assess their state of maturity, individuals were narcotized in 5% ethanol in seawater and a small incision was then made in the lower edge of the 16th setiger. For microscopic analysis, a small sample of coelomic fluid and blood vessel was removed using a 20-μl glass micropipette. The diameters of about 30 oocytes from five randomly chosen females from each treatment group were measured using a compound microscope. Sperm activity was assessed after dilution.

Experimental protocol. On 9 February 1999, half of the females were transferred to conditions of 16 h light and 8 h dark (LD16:8) at 10°C. The individuals that remained in LD8:16 conditions were termed LD8:16 controls while those transferred to the LD16:8 were termed LD16:8 controls. Oocyte diameters were measured from five randomly selected individuals from the two groups on this day and subsequently once per week for 3 weeks.

Prostomium (PM) transplants were performed on 26 February 1999, 2 weeks after photoperiod manipulation had commenced. Individuals from the LD16:8 control and the LD8:16 control were narcotized as described above to provide prostomia. They were termed LD16:8 donors and LD8:16 donors, respectively. The PM was accessed by removing the first two scales and then excised with iridectomy scissors. Once removed, the PM was trimmed of excess flesh and tentacles and then placed in seawater on ice until implanted. Both sets of PM donors were then returned to seawater to recover from the narcotization.

Individuals designated to received the implanted PM were also narcotized, and a PM was inserted through an incision in the 20th setiger. This was far enough from the head to prevent any interference with movement. Twenty-five LD16:8 control females were used as PM donors and their PMs were implanted into females from the LD8:16 control group. They were termed LD8:16$^{PM(LD16:8)}$ females (the superscript notation refers to the conditions that the female, from which the prostomium used for implantation was removed, was exposed to). As a control, eight LD8:16 control females were also implanted with LD8:16 control PMs, and these were termed LD8:16$^{PM(LD8:16)}$. After implantation, all PM recipients and PM donors were returned to their respective photoperiod treatments. All individuals were examined daily for evidence of spawning (the presence of oocytes under the elytra). The diameters of about 30 oocytes were measured from individuals that had spawned.

Statistical analyses. The nature of the oocyte diameter data (unbalanced nesting and sample sizes) precluded analysis using a multiple ANOVA for all data. Instead, mean oocyte diameters for each female were obtained, and these data were then assessed using one-way and two-way ANOVAS. Subsequent pairwise comparisons were performed using Tukey tests. The numbers of spawning individuals were analyzed for independence by using an $R \times C$ contingency table and the chi-square statistic (χ^2); pairwise comparisons were performed subsequently using a modified Tukey test.

Results: photoperiodic control of oocyte growth. Mean oocyte diameters of LD8:16 and LD16:8 controls and PM

recipients (LD8:16$^{PM(LD8:16)}$ and LD8:16$^{PM(LD16:8)}$ are shown in Figure 1. Analysis, using a two-way ANOVA, of the mean oocyte diameters of the LD8:16 and LD16:8 controls for weeks 1 to 3 shows that significant differences were present between weeks ($F = 28.64$, $P < 0.001$), but not between treatments. There were also significant interaction effects ($F = 7.407$, $0.05 > P > 0.001$). Pairwise comparisons using a Tukey test confirm that the mean diameters of LD16:8 and LD8:16 controls were not significantly different from each other in week 1. By week 2, LD16:8 control diameters increased significantly when compared to week 1, whereas LD8:16 control diameters did not increase. By week 3, LD8:16 control diameters increased significantly when compared to week 2. At the same time, all mean oocyte diameters of all treatments were not significantly different from each other when analyzed with a one-way ANOVA. ($F = 0.11$, $P > 0.05$). Analysis of all treatments in week 4 shows that there were significant differences between the treatments ($F = 9.56$, $0.05 > P > 0.001$). However, pairwise comparisons reveal that only the LD16:8 control mean diameter was significantly greater than the LD8:16 control and LD8:16$^{PM(LD8:16)}$. No other pairwise comparisons were significantly different.

Results: photoperiodic control of female spawning mediated by the endocrine system. During the experimental period, all individuals in all treatments were monitored daily for spawning; the cumulative percentage of individual spawning females is shown in Figure 2. Over the duration of the total experimental period (9 February-14 March), 96% (24 individuals) of the LD16:8 controls spawned, with the majority (16 individuals) spawning on 23 February and another 8 spawning between 24 and 28 February. In comparison, only 33% of the LD8:16 control females spawned during the experimental period, one on 19 February, two on 23 February, and another on 25 February.

During the experimental period for prostomial manipulation (26 February–14 March), 80% of the LD8:16$^{PM(LD16:8)}$ treatment group spawned, with 10 individuals spawning on

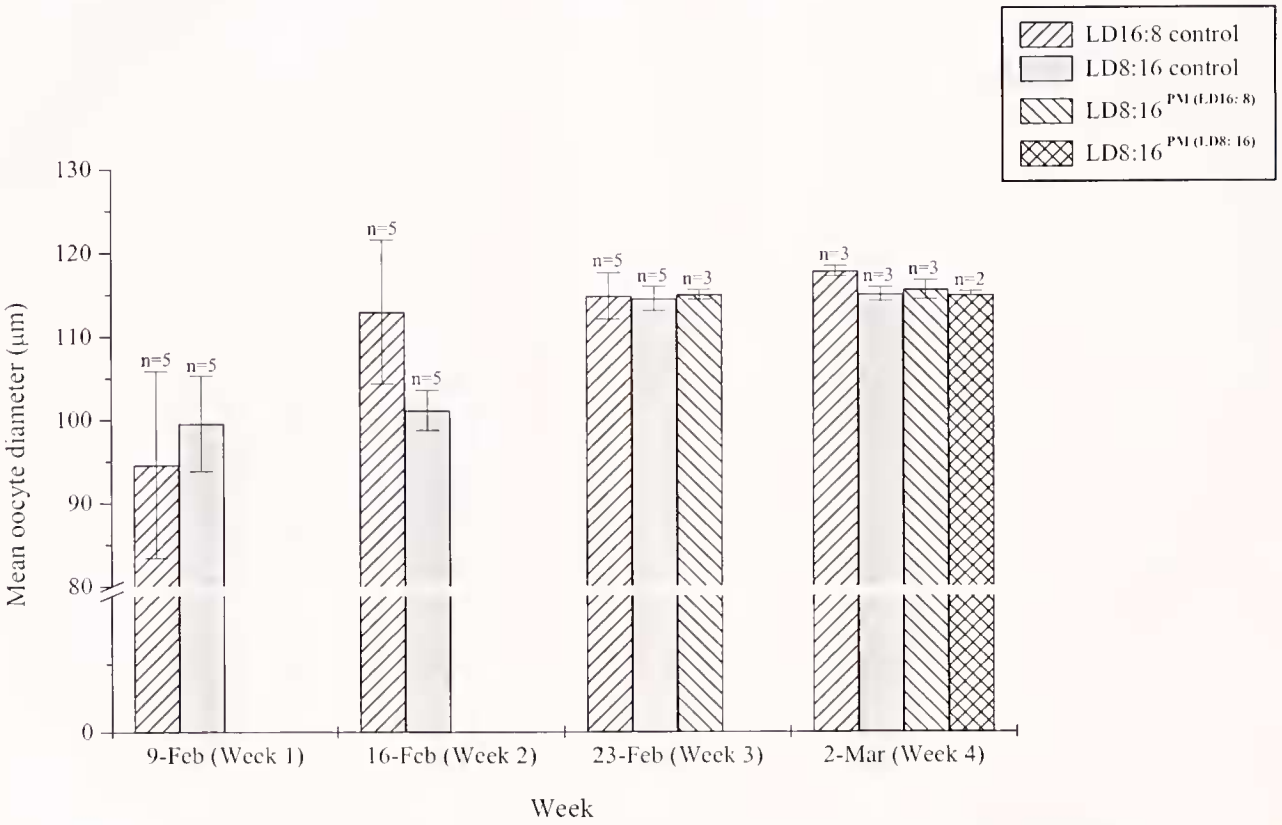

Figure 1. Mean weekly oocyte diameter (±95% confidence limits) of female *Harmothoe imbricata* exposed to various treatments at 10°C: LD16:8 control, females maintained in long-day photoperiod (LD16:8); LD8:16 control, females maintained in short-day photoperiod (LD8:16); LD8:16$^{PM(LD16:8)}$, females maintained in LD8:16, each implanted with one prostomium from an LD16:8 control female; LD8:16$^{PM(LD8:16)}$, females maintained in LD8:16, each implanted with one prostomium from an LD8:16 control female. All individuals were collected in December and maintained in ambient photoperiod at 10°C prior to the start of the experiment on 9 February 1999 (week 1). All transplantations were performed on 26 February 1999 (week 3); n = number of females sampled, 30 oocytes were counted per female.

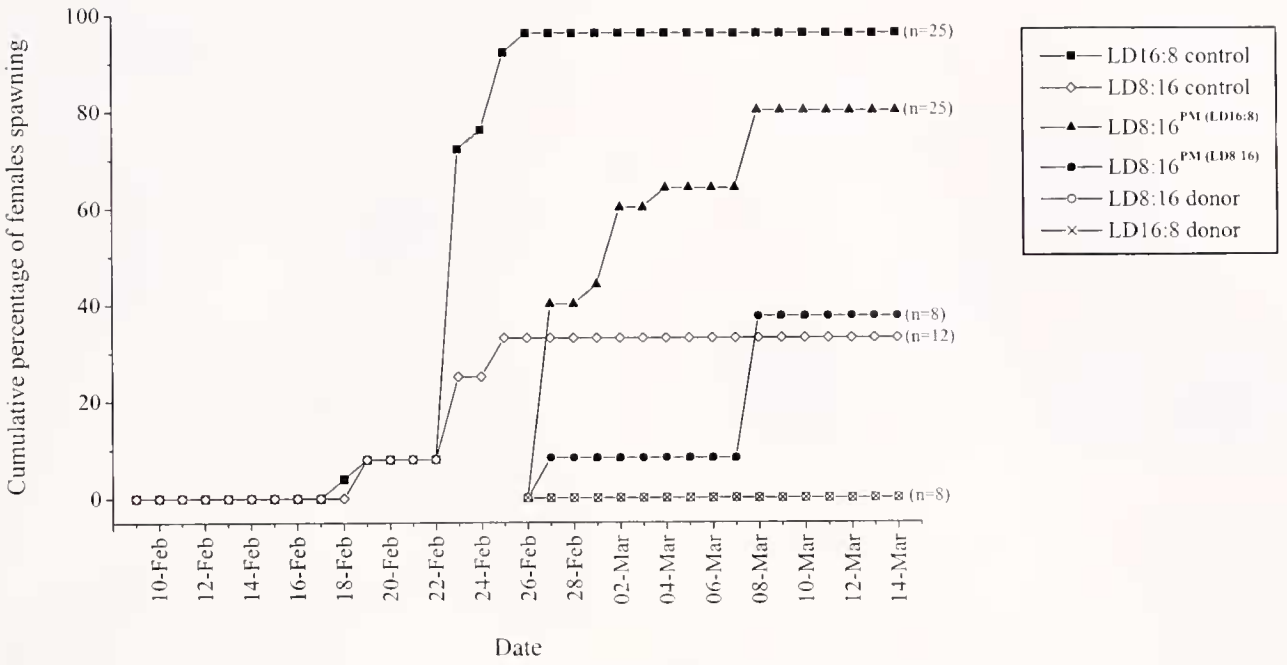

Figure 2. Cumulative percentage of spawning female *Harmothoe imbricata* after exposure to various treatments at 10°C. LD16:8 control, females maintained in long-day photoperiod (LD16:8); LD8:16 control, females maintained in short-day photoperiod (LD8:16); LD8:16$^{PM(LD16:8)}$, females maintained in LD8:16 and each implanted with one prostomium from an LD16:8 control female; LD8:16$^{PM(LD8:16)}$, females maintained in LD8:16 and each implanted with one prostomium from an LD8:16 control female; LD8:16 donor, LD8:16 control females used as prostomial donors; LD16:8 donor, LD16:8 control females used as prostomial donors. All individuals were collected in December and maintained in ambient photoperiod prior to the start of the experiment on 9 February 1999, start of photoperiodic conditioning. All transplantations were performed on 26 February 1999. *n* is the number of females in each treatment group.

27 February and a further 10 spawning between 1–8 March. In contrast, only 37.5% (3 individuals) of the LD8:16$^{PM(LD8:16)}$ treatment group spawned, and none of the LD8:16 or LD16:8 donors spawned.

Statistical analysis using an R × C test of independence and the χ^2 statistic reveals highly significant differences between the total proportion of spawning to nonspawning females between all treatments ($\chi^2 = 68.409$, $P < 0.001$). Statistical analysis of pairwise comparisons, using a modified Tukey test, shows that all pairwise comparisons were significantly different from each other, except the following treatments: LD8:16 control compared with LD8:16$^{PM(LD8:16)}$, and LD8:16 donor compared with LD16:8 donor.

Pheromone influence on pairing behavior

Y-maze behavioral bioassays. Six Y-mazes, the dimensions of which are shown in Figure 3A, were constructed from 5-mm-thick clear acrylic plastic. Each was sealed with silicon sealant and allowed to cure by soaking in seawater for several days prior to use. Each arm had a removable acrylic partition with 0.4-mm-diameter holes drilled through to allow pheromonal diffusion. All experiments were performed at 10°C in ambient illumination.

Animals. Animals were collected from the Fife Ness site during March and April and maintained as described above. Individuals collected in March and April (classed as "old" and "new," respectively) were sexed, and their state of maturity assessed as described above. Of the old individuals collected, 10 females were carrying fertilized oocytes and 7 females still had fully grown oocytes in the coelomic cavity. Of the males collected, 18 had active sperm in their coelomic cavity. Of the new individuals collected, 10 females had not spawned and 10 males had sperm in their coelomic fluid.

Experimental protocol. The basic experimental protocol is summarized in Figure 3B. Before each set of experiments, each maze was washed in fresh water before air-drying. During a run of experiments each maze was washed in TFSW between tests. After each maze was filled, a potential stimulus animal was placed in one arm of the maze behind the partition. After 1 min, a test male was placed in the base of the maze. To minimize handling, a glass tube was used in positioning the test animals. Each test was run for 30 min (except the first directional bias test with old males, see below), after which the position of the test male was noted. At the end of the 30 min, a response was considered positive if the test male was in the arm with the stimulus animal,

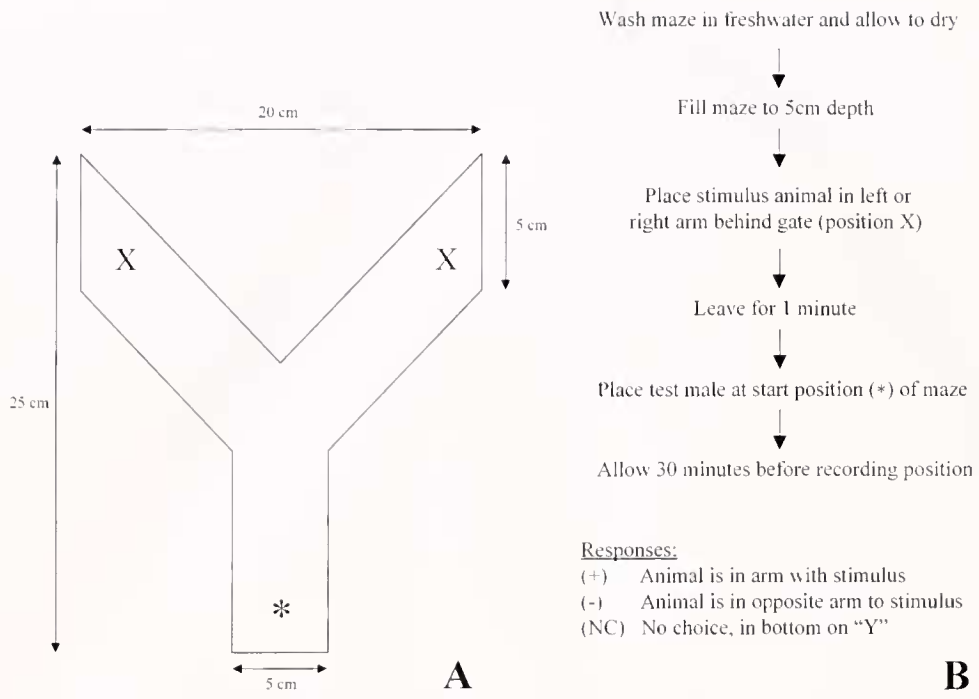

Figure 3. (A) Schematic diagram of Y-maze for the investigation of pheromonal attraction of male *Harmothoe imbricata* to various stimuli. Dashed lines represent removable partitions. Depth of seawater in Y-maze: 5 cm. (B) Summary flow diagram of the experimental protocol.

negative if it was in the opposite arm, and a no choice if it was in the base of the Y. During an experimental run, all potential stimulus individuals were alternated between each arm in consecutive experiments and cross-tested with all test males.

Tests performed. All tests were performed within 2 weeks of collection. To assess for any directional bias ("handedness") and for chance levels of attraction in the maze, two sets of experiments were performed in which males were placed in the Y-maze with no stimulus animal. In the first experiment, nine old males were each run three times in the Y-maze and the position of the animal was recorded after 10 min. From observations of the males, it was suspected that this time period was not sufficient for the test animals to complete exploratory behavior before settling. A further six old males were tested without a stimulus six times each, and their position was recorded after 30 min. All subsequent tests were run for 30 min. Old males were tested against each of the following: old mature females (two sets) and old females carrying fertilized oocytes. New males were tested against each of the following: new mature females, new females maintained in short days, and old males. All statistical significance was assessed using χ^2 analyses.

Results. The first set of experiments was performed with old (collected in March) males and females (Fig. 4). To assess for any directional bias and for chance levels of attraction, the positions of the males were recorded 10 min after being introduced into the maze. Fifty-three percent of

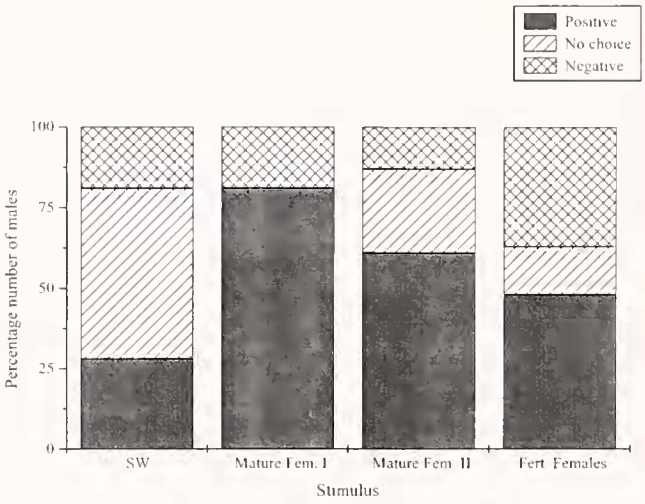

Figure 4. Percentage response of old (collected March 1999) male *Harmothoe imbricata* to various stimuli in a Y-maze run at 10°C. SW, no stimuli added (number of runs = 36 [6 males × 6 tests]); Mature Fem. I, mature old females (number of runs = 27 [9 males × 3 females]); Mature Fem. II, second group of mature old females (number of runs = 63 [7 males × 9 females]); Fert. Females, females carrying fertilized oocytes under their elytra (number of runs = 27 [9 males × 3 females]). The position of each male was recorded 30 min after introduction to the maze. Responses were classed as positive if the male was in the arm containing the stimulus (or designated as stimulus if seawater only), negative if in the arm with no stimulus, or no choice if it moved to neither arm.

these males moved to the arbitrarily labeled positive arm and 47% moved to the negative arm. However, this experiment was omitted from the graph and from statistical analysis because the males had not completed their exploratory behavior within the 10-min time frame. The experiment was, therefore, repeated with a further six old males whose positions were recorded 30 min after introduction. In the 36 tests performed, 28% of the males moved to the arbitrarily positive arm and 19% to the negative arm, while 53% made no choice. This result was not significantly different form an expected value of 33.3% moving to each arm or making no choice ($\chi^2(2) = 2.60$, $P > 0.05$).

These nine old males were then tested against three old mature females, and a significantly higher level of attraction ($\chi^2(2) = 23.07$, $P < 0.001$) was observed: 81% of the males moved to the positive arm and the other 19% moved to the negative arm (without the females). To confirm this attraction response, a further test was performed with 11 males and 7 old mature females. Sixty-one percent of the males moved to the positive arm, 13% moved to the negative arm, and 26% made no choice. This level of attraction was also significantly higher than shown for the seawater 30-min control ($\chi^2(2) = 10.58$, $0.05 > P > 0.001$).

An additional experimental run was performed using the nine old males and three old females that had spawned in the field and were carrying fertilized oocytes under their elytra. In the 27 tests performed, 48% of the males moved to the positive stimulus arm, 37% moved to the negative arm, and 15% made no choice. Statistical analysis of this data shows that this was a significantly higher level of attraction than shown for the seawater control ($\chi^2(2) = 9.69$, $0.05 > P > 0.001$). However, if the no choice and negative results are combined and compared statistically with the positive stimulus, there was no significant difference between the treatments ($\chi^2(1) = 2.74$, $P > 0.05$), confirming that the differences were due to the decrease in no choices and not to an increase in positive results. Statistical analysis also shows that there was a significantly lower level of attraction for fertilized females than for both mature female I ($\chi^2(2) = 6.87$, $0.05 > P > 0.001$) and mature female II experiments ($\chi^2(2) = 7.97$, $0.05 > P > 0.001$).

The second set of experiments was performed with new animals collected in April (Fig. 5). To assess again for directional bias, six new males were tested with no stimulus, and their position was recorded after 30 min. Thirty-three percent and 36% of the males moved to the arbitrary positive and negative arms respectively, with 31% making no choice. This was not significantly different from an expected value of 33.3% moving to each arm or making no choice ($\chi^2(2) = 0.593$, $P > 0.05$).

These new males were then tested against six new mature females. A significantly higher level of attraction was observed when compared to the seawater test, with 61% of the males moving to the positive arm, 22% to the negative arm,

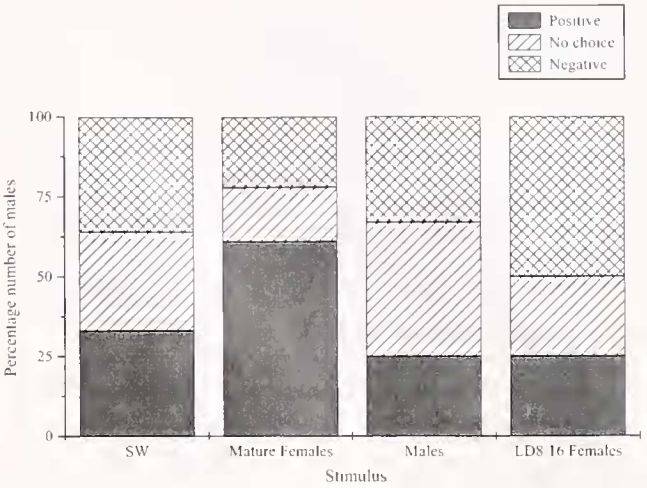

Figure 5. Percentage response of new (collected April 1999) male *Harmothoe imbricata* to various stimuli in a Y-maze run at 10°C. SW, no stimuli added (number of runs = 36 [6 males × 6 tests]); Mature Females, mature new females (number of runs = 36 [6 males × 6 females]); Males: new males tested against themselves (number of runs = 36 [6 males × 6 males]); LD8:16 Females, females maintained in short days (LD8:16) for 3 months (number of runs = 36 [6 males × 6 females]). The position of each male was recorded 30 min after introduction into the maze. Responses were classed as positive if the male was in the arm containing the stimulus (or designated as stimulus if seawater only), negative if in the arm with no stimulus, or no choice if it moved to neither arm.

and 17% making no choice ($\chi^2(2) = 12.95$, $0.05 > P > 0.001$).

These six new males were also tested against themselves. Twenty-five percent moved to the positive arm, 33% moved to the negative arm, and 42% made no choice. Statistical analysis of these data shows that this was not a significantly different level of attraction when compared to the seawater control ($\chi^2(2) = 0.506$, $P > 0.05$), and males were significantly less attractive than mature females ($\chi^2(2) = 12.61$, $0.05 > P > 0.01$).

These males were also tested against six mature females that had been maintained in LD8:16 cycles for 3 months. Twenty-five percent moved to the positive arm, 50% moved to the negative arm, and 25% made no choice. Levels of attraction for this test also did not differ significantly from seawater ($\chi^2(2) = 0.7$, $P > 0.05$), and LD8:16 females were significantly less attractive than mature females ($\chi^2(2) = 10.5$, $0.05 > P > 0.001$).

Discussion

Photoperiodic control of oocyte growth

The rate of oogenesis of the first oocyte cohort in *Harmothoe imbricata* can be altered by manipulation of temperature and photoperiod (Garwood, 1980; Garwood and Olive, 1982; Clark, 1988). A period of exposure of 42–55 days of less than 13 h (winter conditions) photophase is

required to prevent the first cohort of oocytes from being aborted. In experiments in this study, females had been maintained in ambient photoperiods prior to the experiments commencing in February. They were, therefore, not exposed to either LD16:8 or LD8:16 conditions until after the critical minimum number of days had been reached, thus preventing resorbtion.

In the field, once females have been exposed to this critical period of LD8:16 cycles, an increasing ambient photophase (after the winter solstice) allows oogenesis to proceed normally. Above a critical photoperiod of 10–11 h photophase, oogenesis is accelerated; however, an increased number of winter condition cycles (between 55 and 73) is required for this response to be exhibited (Garwood, 1980; Garwood and Olive, 1982; Clark, 1988). The results presented in Figure 1 confirm that an increase in photoperiod (LD16:8 conditions) accelerates oogenesis. The results also show that the response to LD16:8 conditions was rapid, with effects occurring within 1 week from exposure. However, oocyte growth continued under LD8:16 conditions, as by 23 February both LD16:8 and LD8:16 conditions had oocytes that were not significantly different from each other.

Incubating prostomium homogenate with oocytes *in vitro* significantly increases their uptake of radiolabeled amino acid and their subsequent protein synthesis (Bentley *et al.*, 1994; Lawrence, 1996). These authors suggested the presence in the prostomium of a gonadotrophic hormone that promotes and controls oogenesis. The manipulation of photoperiod and temperature may, therefore, be acting directly on the levels of this hormone and it is this hormone level that subsequently mediates oogenesis. Ambient conditions (increasing photophases) in January and February may sustain oogenesis by increasing the circulating titers of this substance. The exposure of females to LD16:8 conditions may have triggered a burst of secretion of the hormone (above the levels normally experienced in February), inducing an increase in mean oocyte diameter that occurred in week 2. Females exposed to continued LD8:16 conditions had no burst of secretion; instead, oogenesis continued at a constant rate, resulting in the delayed increase (week 3) in mean oocyte diameter.

By week 3, oocytes were fully grown—approximately 120 μm (Daly, 1972); data from Figure 1 show that, in these experiments, oogenesis was complete by 23 February. Data also show that implantation of prostomia had no effect on oocyte diameter and did not induce oocyte degeneration. The diameters of spawned oocytes from females implanted with LD8:16 or LD16:8 prostomia were not significantly different from each other, from the LD8:16 controls in weeks 3 and 4, or from the LD16:8 control in week 3. In week 4, the mean oocyte diameters of LD16:8 controls were significantly higher than those in any other treatment, although this is more likely to be due to the low numbers of females sampled than to any effect of the treatment.

Photoperiodic control of spawning mediated by the endocrine system

Early transition through the critical photoperiod can advance the time of spawning; long days or photoperiods with greater than 11 h photophase can cause the first cohort of oocytes to be spawned about 1 month earlier than the natural date (Garwood and Olive, 1982). In this study, females carrying fertilized oocytes were collected from the field on 3 March 1999. We calculated that their natural spawning data was late February to early March. From the results presented in Figure 2, exposure to a LD16:8 photoperiod did not induce notably earlier spawning dates when compared to a LD8:16 photoperiod, and neither was different from the natural date. Instead, when compared to LD16:8 exposure, LD8:16 exposure actually prevented spawning in the majority of females. Ninety-six percent of the LD16:8 females spawned, as opposed to only 33% of the LD8:16 females.

We conclude that the presence of the prostomium is required for spawning to occur in *H. imbricata*, as none of the females that were used as LD16:8 or LD8:16 donors spawned. Examination of the oocytes form these donors also showed that they had not increased in diameter after prostomium removal and had begun to degenerate (data not shown). The prostomium is, therefore, required for the maintenance of oogenesis and for spawning to occur. Nevertheless, a failure to spawn after prostomium removal cannot, at present, be attributed solely to a loss of endocrine function because it may also be due to the severance of nervous connections controlling spawning.

To investigate whether the inhibition of spawning through exposure to LD8:16 photoperiods is endocrine mediated, we implanted LD8:16 control females with prostomia from females maintained in LD16:8 or LD8:16 photoperiods for 2 weeks. Although the implantation of prostomia is an established technique for investigating the role of endocrine substances in polychaetes (see Golding, 1987), this is the first time that it has been used successfully for *H. imbricata*. Previous attempts with this species resulted in the degeneration of the implanted prostomium (P. J. W. Olive, University of Newcastle upon Tyne, pers. comm.). Prostomia implanted during our experiments showed no obvious degeneration with light microscope analysis up to one month after implantation (data not shown). It should be noted that implantation of prostomia is not an ideal technique for identifying the putative spawning hormone in this species. Future investigations will focus on the development of *in vitro* bioassays to reduce the numbers of prostomia used and to meet the levels of sensitivity and reliability that are essential for purification studies.

The results presented in Figure 2 show that 80% of the LD8:16$^{PM(LD16:8)}$ females spawned, as opposed to 37.5% of the LD8:16$^{PM(LD8:16)}$ females. These results confirm that the

implanted prostomia were still functioning as endocrine organs. We suggest the following hypothesis for the endocrine control of spawning. A spawning substance present in the prostomium is required for spawning to occur. The titers of this substance, as with the gonadotrophic hormone, are mediated by photoperiod. The exposure of females to lengthening photophases (ambient conditions in February) increases the titers of this spawning substance to a level above which spawning can occur. Exposure of females to LD16:8 conditions also allows titers of the spawning substance to reach the threshold level, so that nearly all the females spawn. In contrast, only a small proportion of the females exposed to LD8:16 conditions have titers of the spawning substance that reach the threshold, so significantly fewer spawn. Implanting the prostomia of an LD16:8 exposed female into an LD8:16 female also provides a source of higher levels of spawning hormone and thus increases the total circulating titers, enabling the LD8:16 females to reach the threshold required for spawning to occur. Implanting the prostomia of an LD8:16 exposed female into an LD8:16 female provides a second source of the hormone, but at lower concentrations. In most females, the combined level of hormone is lower than the threshold, so most fail to spawn.

The nature and action of a spawning substance in *H. imbricata* may take two forms. It could be a "true" spawning substance like that found in *Nephtys hombergii.* In that species, a hormone released from the supraesophageal ganglion induces spawning by acting on the musculature to allow the release of gametes through the anus (Bentley *et al.*, 1984). The maturation of the gametes is independent of the spawning hormone; they mature once released into seawater (Olive, 1976; Olive and Bentley, 1980).

The other form of spawning substance induces gamete maturation and subsequent spawning either directly or indirectly. This form occurs in *Arenicola marina.* Oocytes mature through a two-step system involving a substance from the prostomium and then a second substance in the coelomic fluid. The latter, termed the coelomic maturation factor (CMF), induces the oocytes to mature; these oocytes are subsequently spawned (Watson and Bentley, 1997). It is unclear whether CMF also acts on the musculature to facilitate spawning. However, in male *A. marina,* the sperm maturation factor (8, 11, 14 eicosatrienoic acid) not only induces maturation of the sperm but also produces specific behavioral changes associated with spawning (Pacey and Bentley, 1992).

In *H. imbricata,* oocytes are released from the ovaries at prophase of the first meiosis a few days before spawning. They mature to metaphase of meiosis I in the coelomic fluid, are collected by the nephridia, and spawned (Daly, 1972). Further experiments are required to elucidate whether the putative spawning substance from the prostomium can induce spawning of immature oocytes or actually induces the maturation of the oocytes that are subsequently spawned.

Pheromone influence on pairing behavior

Initial observations of the test males in the Y-maze indicated that 10 min was not sufficient time for them to complete their exploratory behavior. After 10 minutes most of the males were still actively searching. In all subsequent experiments we allowed 30 min for the males to settle before their position was recorded. This time period was sufficient for the males to settle and complete their exploratory behavior.

Results presented in Figures 4 and 5 provide the first evidence that pheromones are involved in the reproductive behavior of *H. imbricata.* Specifically, these data indicate that a mature female with fully grown oocytes in its coelomic cavity releases a waterborne substance or substances that attracts significantly more mature males than are attracted by seawater, males, or females that are carrying fertilized oocytes.

Harmothoe imbricata is a solitary species outside the breeding season, but it reproduces by forming single copulating pairs (Daly, 1972). This method of reproduction requires mature individuals to locate each other, but at low population densities, chance encounters may be infrequent. An attraction pheromone released by a mature female increases the chances that a male will find her and, therefore, increases the number of successful fertilizations. The pheromone may also maintain the pair bond and could suppress the cannibalistic tendencies of both individuals, allowing pairing to proceed. Once the female has fertilized oocytes, she stops releasing the pheromone and becomes unattractive to the male; this is confirmed by the data shown in Figure 5.

One of the best-studied attraction pheromones in marine invertebrates is attractin, a peptide found in the egg cordon of the opisthobranch mollusc *Aplysia* spp. The function of this 58-residue peptide is to attract other individuals to the mating aggregation and to induce mating (Painter *et al.*, 1991, 1998). Pheromones are also a component of a number of polychaete reproductive strategies, particularly in some nereid species (for review, see Zeeck *et al.*, 1996). However, these pheromones have been isolated only from species such as *Platynereis dumerlii* and *Nereis succinea* that swarm *en masse* in the water column (Zeeck *et al.*, 1988, 1996; Hardege *et al.*, 1998). The results presented here are, therefore, the first report of a waterborne cue being used as an attraction pheromone from a polychaete that reproduces following pair formation.

Although the evidence for pheromones in marine invertebrates is steadily growing, the environmental control of pheromone production has not been investigated. The effect of environmental manipulation (particularly photoperiod) on oogenesis is confirmed and the influence of photoperiod

on spawning in *Harmothoe imbricata* has been described for the first time (Garwood and Olive, 1982; Clark, 1988). Establishing a link between the photoperiodic input and the production of a pheromone that regulates spawning behavior is an important step. Results presented in Figure 5 show that females maintained in LD8:16 photoperiods were only as attractive to mature males as seawater or other males. Just as LD8:16 exposure may prevent the production (or the attainment of threshold levels) of the putative spawning hormone and thus prevent spawning, it may also prevent the production of the attraction pheromone. The relationship between the spawning hormone and pheromone production requires further investigation, but the production of the two may be intricately linked, and they may be the same or similar substances.

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